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SOME LABORATORY METHODS IN EMBRYOLOGY.

BY R. G. HOSKINS.

THIS paper is a description of some of the methods employed in the zoology department of the University of Kansas in the preparation and handling of material for routine work in embryology. There is not much herein that is original; it is rather a systematic arrangement of data adapted from many sources. The methods have this, however, to recommend them: they have all been found satisfactory under ordinary working conditions. The material used is mostly as follows: For holoblastic cleavages of the egg, the frog; for delamination and early organogenesis, the chick; and for later organogenesis, the pig. The method of preparing each for class use will first be given.

Frog eggs are collected in early May, in a rather extensive series of lots. From these are selected, if possible, the various stages desired. If any stages are lacking, a lot of younger age is kept in a dish of water exposed to bright sunlight until the right stage of development is reached. They are then preserved in four per cent. formalin until wanted. The stages used are 1-, 2-, 4-, 8-, 32- and 128-cell, early and late blastopore, and a stage showing the neural folds.

For chick material, a satisfactory supply of eggs can usually be obtained from some farmer without any special trouble. It is well, however, to get them in the spring, when the vitality of the eggs is strongest. These are placed in an incubator for the necessary time to secure embryos of any desired age. In reckoning the time to remove the eggs, one hour should be allowed in addition to that required for the desired develop-

ment, as it takes about that length of time for incubation to begin. The embryos thus secured are not of exactly uniform age, but a count of the number of somites affords a satisfactory check for chicks of from twenty to fifty hours, and the general appearance of the younger stages is characteristic; ordinarily, for stages older than fifty hours, the incidental variation in immaterial. In this laboratory the "Cyphers" hot-air incubator has proved very satisfactory. During incubation the eggs should be turned once or twice a day to prevent adhesions between the fetal and shell membranes.

After sufficient incubation the eggs are removed and the embryos secured by the following method: The shell is perforated at one side so as to avoid injury to the chick, which normally floats on the top. Then the shell is carefully removed, bit by bit, until a hole about the size of a five-cent piece is made. For this purpose a pair of curved forceps and a needle with a sharply recurved point are very convenient. The chick is now fixed *in situ*. This is much simpler and more satisfactory than the process frequently employed of floating out the egg in normal salt solution. Incidentally, however, this later process can occasionally be employed to advantage, as a last resort, in case of an embryo that is not normally oriented. The fixative used must be one that does not cause an adhesion between the vitelline membrane of the egg and the embryo. The following mixture has been found most satisfactory:

FORMO-NITRIC.

Ten per cent. formalin	3 parts.
Ten per cent. nitric acid	1 part.

This fluid requires from five to fifteen minutes for the fixation of embryos up to seventy-two hours old. For older chicks a subsequent treatment in this or some other good fixative is advisable.

After fixation is completed a disc including the embryo is cut out from the vitelline membrane by means of a pair of curved-pointed scissors. This disc for embryos up to twenty-four hours old should be about the size of a dime; then up to thirty-six hours of age the incision should follow the margins of the blastodisc, just outside of it; above this age any

desired amount of the fetal membranes may be included. The disc is now removed on a section-lifter to a Petri dish of water. By gentle manipulations the adherent yolk and the vitelline membrane can be floated loose from the embryo. In case they prove refractory a gentle agitation of the water by a pipette will usually dispose of the difficulty. At this stage great care is necessary.

For convenience in further manipulation it is advisable to harden the embryos, particularly if they are to be sectioned. They are carefully flattened in a vessel with a plane bottom —preferably a Petri dish—then treated with the hardening fluid—ten per cent. formalin saturated with mercuric chlorid. Only a few drops should be used, and this carefully applied, in order to avoid any folding or creasing of the blastodisc or membranes. After a treatment of from five to thirty minutes, the embryo is washed in running water from fifteen minutes to two hours—the length of time depending on the size. This step follows immediately after fixation, in case the hardener is not used.

For staining surface mounts for general microscopic study, Grenacher's alum-carmine is very satisfactory, but if the preparation is to be used with a projection lantern, borax-carmine is slightly better. For embryos that are to be sectioned, either of these may be used as an *in toto* stain, but it is often advisable to use others, staining on the slide. In this case the chick should be given an *in toto* stain with some such color as eosin; this makes it more easily seen, and hence less likely to injury in the manipulations of sectioning and mounting. This stain is later removed from the sections with alcohol and some other stain substituted. Mayer's hæmalum is perhaps the best for general purposes. Older stages should be stained very lightly. For clearing whole embryo preparations pure cassia oil has been found much superior to anything else; it causes very little shrinkage and does not make the material brittle, as do most other clearing agents.

To secure pig material in any considerable amount, it is necessary to visit a large pork-packing establishment. In any of these it is easy to secure embryos of any size from

5 mm. up to full term, and in any desired quantity. For this purpose it is sometimes recommended that a room be secured in or near the packing-house, where the pregnant uteri can be taken for examination. Unless, however, very small embryos are desired, this will not be found necessary. It is better to obtain a table in the cleaning room as close as possible to where the uteri are removed. This permits the handling of the material with much greater dispatch, with sufficient convenience for all practical purposes, and with little, if any, greater amount of incidental unpleasantness.

For equipment, the collector needs only a good supply of containers, a small graduate, fixatives, and, unless he is desirous of some special kind of material, one pair of small scissors; anything more is only in the way. For fixation, Kleinenberg's picro-sulfuric mixture has been found quite satisfactory for embryos up to 10 or 15 mm. long and Zenker's fluid for all sizes. The larger ones, however, must have the body cavity punctured to secure sufficient penetration of the fixative. Where the size of the embryo does not preclude its use, the former is the more satisfactory, both on account of the simplicity of the after-treatment required, and on account of the greater length of time material can be left in it. Later stages, for the purpose of dissection, are best preserved in Erlichi's mixture:

Potassium dichromate	2½ gr.
Cupric sulfate	1 gr.
Water	100 cc.

Containers of standard size should be used and the dry materials of the fixatives should be weighed out and carried in vials with sufficient amounts in each to make one container of fluid. It is more practicable, however, to carry the liquid ingredients in bulk and measure them out as needed. The mixing of the fixatives is thus very simple, since the only other ingredient, water, is always, in a cleaning room, near at hand. In case Zenker's fluid is used, enough Müller's should be taken to give each embryo a preliminary bath to fix the albuminates, which would otherwise impede the action of the former fluid.

The pregnant uteri can be distinguished by their greater

vascularity; also, in case of those containing embryos of greater length than 5 or 6 mm., by the series of enlargements that contain the latter. These uteri are selected and carried to the table for examination. Each of the previously mentioned enlargements is then slit open with the scissors. This will also slit the false amnion, thus opening the vesicle in which floats, in a copious supply of fluid, the true amnion and its contained embryo. This is then seized and the false amnion, to which it is attached, severed with the scissors. For handling the embryos at this stage of the manipulations, fingers are preferable to any sort of forceps. After a sufficient number of embryos have been obtained and placed in the fixative, they are taken to the laboratory and, after fixation is completed, washed, sorted, and preserved in eighty per cent. alcohol until needed.

As in case of the chicks, the pig embryos to be sectioned should be given a preliminary stain with eosin before they are embedded. They are then sectioned at a thickness of from 10 to 20 microns, depending upon the size of the embryo. The sections are mounted on slides and the eosin removed. For staining, the most satisfactory results have been obtained by the following hæmalum-cochineal mixture: Equal parts of Minot's alum-cochineal and Mayer's hæmalum; this is followed by a counterstain of orange G. This method can be used with material fixed either in Zenker or picro-sulfuric. With this combination there is very little danger of overstaining; the cochineal has a retarding effect on the hæmalum, and finally so impregnates the tissue as to prevent overstaining with the latter. An excess of orange G can be easily corrected by an alcohol bath. This combination gives exceptionally good differentiation; the cochineal is selective for nervous tissue and brings out the various elements in the central nervous system particularly well, while the hæmalum gives a very clear nuclear stain. The orange G marks off distinctly both cell and basement membranes, as well as peripheral nerve fibers.

Each mounted embryo that is added to the collection is given an accession number. This is done whether it is put up as an exhibition specimen, either whole or dissected, or

sectioned and mounted on slides. In the latter case, each slide bears in the upper right-hand corner the accession number of the embryo and the number of the slide in the series. (Thus the fifth slide of embryo No. 70 would be marked $\frac{70}{5}$.) This marking is done permanently with a diamond point; this obviates the annoyance incident to the loss of paper labels; these can, however, be used in addition to the numbers, with advantage in some cases. A record of each embryo and slide is kept by a card catalogue system. For this purpose a set of cards has been devised by Mr. R. E. Scammon, of this department. He, however, expects to describe these in a separate paper.

For class use, any series can be divided up into sets as desired, as brain sets, heart sets, etc. For containers of these sets, the flat, pressed-board boxes manufactured by Theodore Schröter, of Leipzig, are very convenient. They can be obtained of various sizes as desired, and have the advantages of compactness and lightness. For storing series of more than six slides, ordinary slide boxes of 100 capacity are used. Standard sets are filed away in the flat containers, but incidental sets are better reassembled in their regular containers when not in use. This system of handling them makes any slide of a collection of any size available for instant reference. It also reduces to a minimum the work of keeping the collection in order, since any "stray" slide can be quickly restored to its place at any time.